

Original Article



In vitro and *in vivo* studies of cytotoxic effects of FeSO₄ nanoparticles

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Abstract

Background and aims: Using iron as a food additive usually causes undesirable sensory changes and side effects in humans. In this study, we made iron (Fe) nanoparticles (NPs) and studied the cytotoxicity of FeSO₄ bulk and NPs on HT-29 cells and different doses of these particles on rat intestine.

Methods: Particle size of nanoscale was achieved by mechanical technique. Iron particles were characterized using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The effect of iron particles with different concentrations (6.25, 3.125, and 1.57 mM/mL) on the colon cell line was performed using the MTT assay at 24, 48, and 72 hours. Apoptosis and necrosis of the cells were assessed using Annexin V-FITC staining and propidium iodide (PI) at 24 h. In an *in vivo* study, Taftoon bread was produced from fortified wheat flour with FeSO₄ bulk and NPs, which are recommended in human diet (9, 18, and 27 mg of elemental iron/kg flour). Wistar rats were fed daily with fortified bread for 21 days and their colon and small intestine were then evaluated histopathologically. Statistical analyses were performed using SPSS 22.0 software by chi-square test.

Results: The synthesized FeSO₄ NPs were smaller than 100 nm, and they had more adverse effects on the viability of the HT-29 cells compared to the bulk- FeSO₄ at 72 hours. Flow cytometric study showed that the early apoptosis of cells by the bulk form was more than the NPs, but at the low concentration (1.57 mM/mL), the NPs induced more necrosis than the bulk particles ($P=0.063$). The survival rate of cells facing all concentrations of NPs and bulk- FeSO₄ decreased dose dependently ($P=0.075$). *In vivo* results revealed that there were no pathological changes in rats' intestinal tissues.

Conclusion: The bulk and NPs of iron have adverse effects on the HT-29 cells, but no histopathological changes were seen on rats' intestinal cells.

Keywords: Iron nanoparticles, Intestinal cell culture, Histopathology, Cytotoxicity

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Introduction

Despite the comprehensive application of nanoparticles (NPs) in foods, such as improving flavour and texture, increasing shelf life, enhancing dietary value, the small size of these nanoparticles, i.e. less than 100 nm, and the possibility of their attachment to vital body structures and causing toxic effects, stay a source of concern among scientists, as reflected in the research undertaken (1-12). Moreover, NPs' properties can affect their toxicity to biological systems such as cell culture (13-15). Therefore, determination of a suitable dose of NPs in cytotoxicity assessments can influence their toxic impacts (16). In addition, global investments in nanotechnology research have dramatically increased (17,18) and numerous cytotoxicity studies are still ongoing to address the

growing concern over the toxic effects of NPs on human health (15,19). As the most abundant metal in the environment and an important and well-known micronutrient, iron is vital for biochemical reactions in the body and for producing blood cells. Iron deficiency is also globally prevalent as the most widespread nutritional disorder (20). In the event that the oral iron supplied is insufficient to make red blood cells, the body first uses its reserves, including the iron stored in the liver, leading to iron deficiency anemia if the condition is not treated. The improper distribution of iron-enriched foods, the delayed beginning of supplementation, the insufficient daily dose of prescribed iron, and malnutrition are factors that contribute substantially to iron deficiency anemia. Today, iron deficiency anemia is a prevalent and

widespread nutritional disorder during pregnancy in most countries, including Iran, and a major risk factor for increasing the maternal and neonatal mortality and the prevalence of low birth weight and premature birth (21-23). Enriching foods such as bread with iron supplements is an effective and inexpensive strategy for controlling and preventing disorders associated with iron deficiency or iron deficiency compensation; nevertheless, this strategy can cause problems such as changing the taste and color of the food or gastrointestinal tract complications (24-26). Iron compounds with the highest bioavailability can cause undesirable sensory changes as they are strongly attracted to nutrient components. The optimal iron compound selected for food enrichment should have the highest relative bioavailability, be cost-effective, and does not cause unacceptable sensory changes in taste, color, and texture of the food. Divalent iron sulfate is a compound with the chemical formula of FeSO_4 , which is widely used as a water-soluble iron enrichment agent for enriching flour owing to its cost-effectiveness. FeSO_4 is a generally recognized as safe (GRAS) compound that is recommended for low-extraction flour enrichment. FeSO_4 salt with the highest bioavailability among iron compounds is a peroxide whose ferrous ions are converted into ferric ions during oxidation. Ferric compounds can change the color of food products into brown, and are less absorbed in the intestine due to its insolubility. Its large particles also cause fairly acceptable darkness in the flour and black spots in the bread (27-30). Given the extensive use of iron as a food additive, the current research aimed to discover data on ways to reduce undesirable sensory modifications and side effects induced by their bulk form by studying the cytotoxicity of nanosized and bulk iron particles to HT-29 cells and the impacts of separate doses of these particles on the intestine of rats.

Materials and Methods

Synthesis of NPs and Characterization of Particles

A planetary ball mill made by Fritsch Pulverisette 6 was used to physically synthesize 87% pure FeSO_4 as NPs. To prepare these NPs, the bulk FeSO_4 was poured into the machine and milled at 200 rph for 15 hours with a ball-to-powder weight ratio of 10:1 (31).

The particle shape, size and mean diameter of NPs was observed by scanning electron microscopy (SEM; KYKY-EM3200, China) and transmission electron microscopy (TEM; Philips CM 10, Eindhoven, The Netherlands).

In vitro evaluation of the toxicity of iron NPs

The human intestinal HT-29 cell line can be a good model for cytotoxicity assessments of different materials for cells (32). The human colon cell (HT-29) line was procured from the National Cell Bank of Pasteur Institute, Tehran, Iran. These cells were grown on RPMI 1640 Medium (Roswell Park Memorial Institute medium, Gibco,

USA) along with 100 U/ml of penicillin, 100 $\mu\text{g}/\text{mL}$ of streptomycin, and 10% fetal bovine serum (FBS, Gibco, USA), and incubated at 37°C in an atmosphere containing 5% CO_2 (33). These cells were cultivated as single layers in a flask. The culture medium was substituted three times a week, and the cells were isolated with trypsinase and passaged at 80%-90% confluence. The cells were ultimately counted and cultured in 96-well plates.

Cell Viability Assay

The MTT assay is a quantitative and staining test based on the reduction of the water-soluble yellow salt of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide and the formation of insoluble dark-blue formazan crystals in water. The MTT reduction occurs only in viable cells by the mitochondrial enzyme of succinate dehydrogenase. The cells viability is therefore measured with the MTT assay. Colon cells (1×10^4 cells/well) were fully grown in 24 hours on the 96-well plates in RPMI 1640 medium. After reaching 80-90% cell confluence, the supernatant was replaced with a new medium containing different doses of nanosized and bulk iron particles, i.e. 6.25, 3.125, and 1.56 mM/mL. The selected doses were based on the previous studies (34,35), and the test was performed with three interactions. After 24, 48, and 72 hours, 10 μL of filter-sterilized MTT (5 mg/mL in PBS, Melford) was added to each well and incubated for 4 hours at 37°C. In the next step, the Formazan crystals produced were dissolved into each well by adding 100 μL of DMSO (Roth, Germany), and the produced stain was read at 570 nm using an ELISA reader. The viability was calculated using the relative absorbance and expressed as the percentage of that of the control sample (36).

Apoptosis and necrotic cells were evaluated by using Annexin V staining and Propidium iodide (PI). The cells were cultivated for 24 hours with distinct levels (6.25, 3.125, and 1.56 mM/mL) of the FeSO_4 bulk and NPs. Cells were detached and stained with PE-Annexin V and PI, based on the manufacturer's protocol of Annexin V apoptosis detection kit I (BD Pharmingen™) and analyzed by flow cytometry (Partec, Germany).

Production of iron-enriched bread

Wheat flour was first obtained from Danesayan Factory™ in Kerman, Iran, and mixed with a certain amount of iron NPs and bulk iron. After adding the yeast (*Saccharomyces cerevisiae*, PTCC 5080), 55%-60% water and 1%-2% salt, bread containing 9, 18, and 27 mg of iron/kg of flour was prepared (37).

In vivo evaluation of the toxicity of iron NPs

Preparation and grouping of animals

A total of 49 Wistar rats (*Rattus norvegicus*) weighing 280 ± 20 g were procured from the national laboratory animal house in Kerman, Iran. The animals were kept in

polyvinyl carbonate cages for one week in an animal house at 22±2°C and humidity of 55±5% and in 12:12 light-dark cycles (38). They had free access to food and water. All the ethical principles of conducting animal research were observed. The rats were then randomly divided into seven groups (n=7 in each), including a control group. Three groups treated daily with bread enriched with bulk iron containing 9, 18, and 27 mg of iron/kg of flour and, three groups treated daily with bread enriched with iron NPs with the same dose for 21 days (39-41). These groups were carefully examined for emerging clinical symptoms. After the period of treatment, rats were euthanized using 80 mg/kg ketamine and 2.5 mg/kg Acepromazine IV and histological samples were taken.

Histopathology

Samples ultimately were taken from the colon and small intestine of the rats, and were fixed with 10% formalin. Hematoxylin and eosin (H&E) stain was used to examine the pathological changes.

Statistical analysis

The data were analyzed in SPSS 22 software by chi-square test, and $P < 0.05$ was considered a significant difference. The data obtained from histopathological studies were also investigated and expressed using descriptive statistics.

Results

Characterization of particles

The particles' morphology was nanospheres, and the mean particle size decreased to less than 100 nm after nanonization (Figure 1).

Cell proliferation and metabolic activity

The results showed that HT-29 cells metabolic activity in all the three concentration, except for 6.25 mM/mL of bulk particles, decreased after 72 hours in a dose- and time-dependent manner, so that cell viability at different concentrations of bulk FeSO₄ (1.57, 3.125, and 6.25 mM/mL) was 100%, 96%, and 73.7% at 72 hours, respectively. However, the survival rate of cells facing NPs was 66.9%, 87.4%, and 59.7%. The cell viability decreased by approximately 40.3% of bulk FeSO₄ at high concentration (6.25mM/mL) in the colon cell line.

Furthermore, the results showed that cell viability decreased from 89.2% to 73.7% and from 77.4% to 59.7% by bulk and NPs, respectively, at high concentration (6.25mM/mL) in a time-dependent manner.

The statistical analyses revealed that there were no significant differences among control and treatment groups ($P = 0.063$). Moreover, no significant difference was seen between groups treated with bulk Fe and Fe NPs ($P = 0.75$) (Figure 2).

Furthermore, flow cytometry determined the amount of necrotic and apoptotic cells. The maximum amount

of necrotic cells was associated with a 1.57 mM/mL concentration of bulk FeSO₄. However, the flow cytometry of the particles found the highest cell viability to be associated with the low concentrations of FeSO₄ NPs, i.e. 1.57 and 3.125 mM/mL (Figures 3 and 4). Therefore, the effect of NPs was more than the bulk particles on the HT-29 cells.

Histopathological Analysis of Rats

The histopathological examination revealed that there were no pathological changes in intestinal cells. Moreover, hemosiderin pigments were not observed in the small intestine and colon tissues of any of the studied samples (Figure 5).

Discussion

In the present study, FeSO₄ NPs were synthesized using a planetary ball mill, and the cytotoxicity of the NPs and bulk iron particles to HT-29 cells was examined using the MTT assay. The effects of different doses of these particles were also studied on the intestine of the rats fed by bread enriched with these particles. The results showed that the viable cells decreased in groups treated with bulk and NPs of iron, but necrosis and apoptosis in cell lines were more in the bulk iron treatment groups than iron NPs.

In 2014, Sahu et al investigated the cytotoxic potential

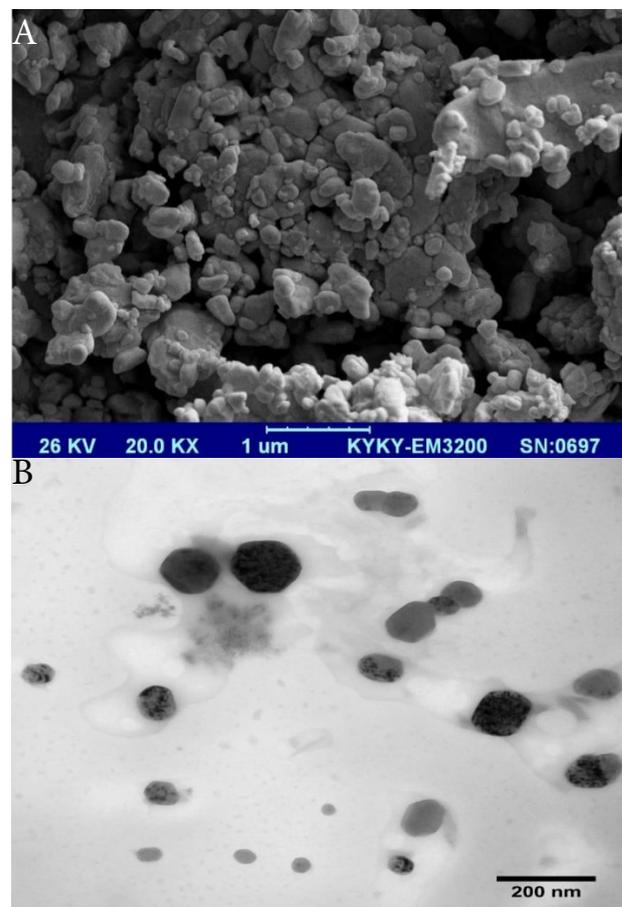


Figure 1. SEM image (A) and TEM image of FeSO₄ NPs (B).

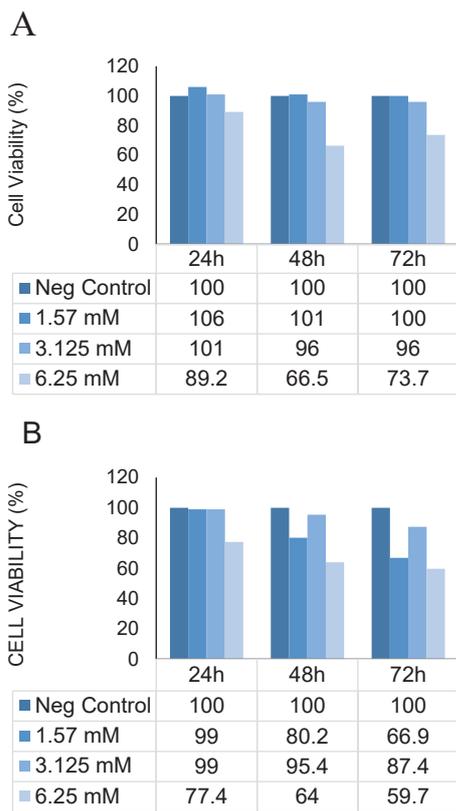


Figure 2. In vitro cell viability assessment of (A) FeSO₄-bulk and (B) FeSO₄-NPs on HT-29 cells after 24, 48, 72 h by MTT assay.

of silver NPs (20 nm in diameter) by studying HepG2 and the human intestinal HT-29 cell line. They found these cell lines to be always a good model for evaluating the toxicity of different materials to cells (32). A dose-dependent viability reduction was observed for 72 hours in HT-29 cells compared to the control group at concentrations of 3.125 and 6.25 of the bulk iron. Zödl et al also observed reductions in the signal in the MTT assay at 1.5 mM/mL of FeSO₄ and an associated reduction in the viability of HT-29 cells compared to FeCl₃ (34). These results are in agreement with the results of the present study, as cell viability decreases with increasing concentration of NPs. The flow cytometry showed that the concentrations 3.125 and 6.25 mM/mL of the bulk iron increase necrosis in intestinal cells after 24 h compared to the Fe NPs. Meanwhile, the viability of cells ultimately decreased at all the concentrations of Fe NPs by increasing the incubation duration to 72 h. The same results were reported by Böhmert et al in 2012, and they found the strong cytotoxic effects of silver NPs on the proliferation and differentiation of HT-29 cells to increase with an increase in the dose and duration of incubation and a decrease in the particle size. Cytotoxicity of the NPs began with causing morphological changes and reducing the cell binding capacity, leading to apoptosis within 24-48 h of exposure (42).

In 2013, Yang et al examined the toxicity of silica NPs

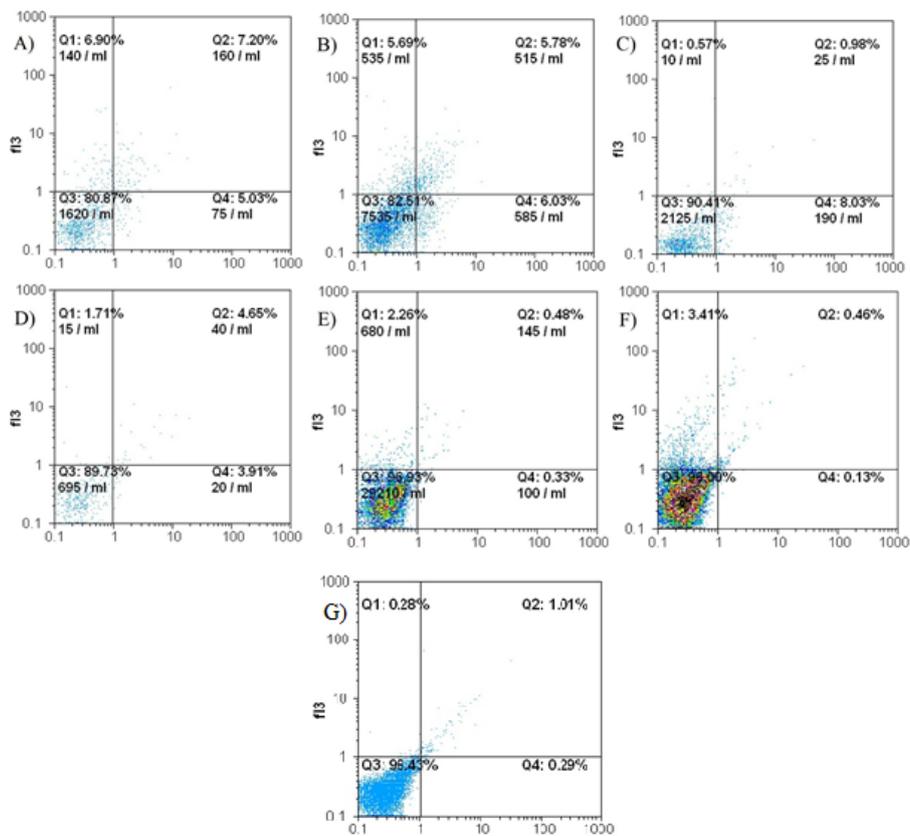


Figure 3. Dot plots of the HT-29 cells apoptotic events induced by FeSO₄ particles after 24 h treatment with: (A, B, C: FeSO₄-bulk, 6.25, 3.125, 1.56 mM/mL respectively; D, E, F: FeSO₄-NPs, 6.25, 3.125, 1.56 mM/mL respectively, and G: Ctrl), where: where Q4: early apoptotic cells, Q3: viable cells, Q2: late apoptotic cells and Q1: necrotic cells.

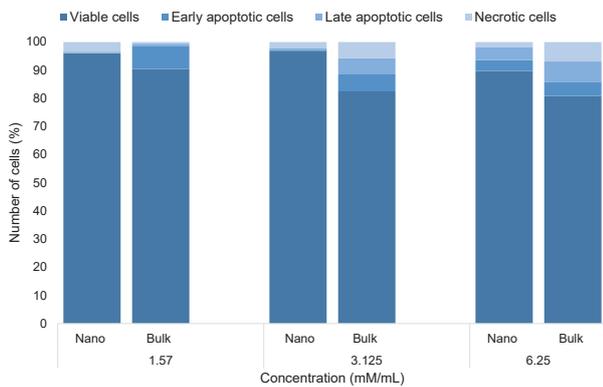


Figure 4. The percentage of HT-29 in terms of living, early apoptotic, late apoptotic, and necrotic cells

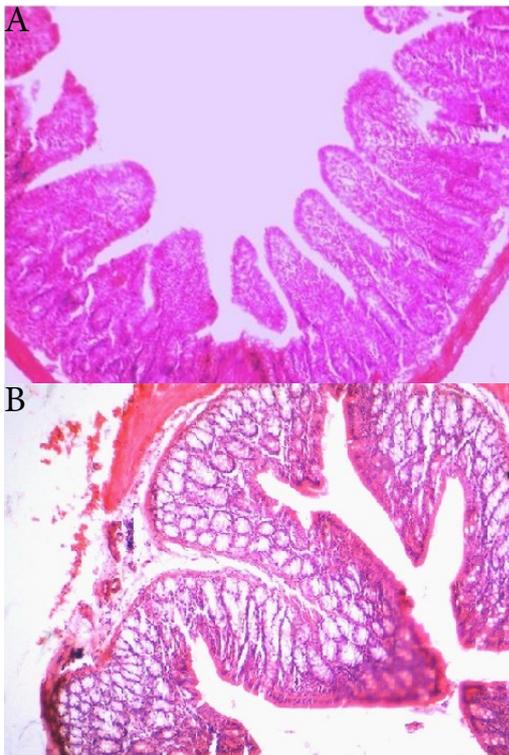


Figure 5. No pathologic lesion was observed in the rat's small intestine (A) and colon (B) (H&E staining, 40X).

as a food additive to cells in the gastrointestinal tract, namely GES1 and HT-29. They produced silica NPs with a maximum concentration of 100 µg and reported that there were no harmful effects on cells in the gastrointestinal tract within 72 hours of exposure. But the concentration of at least 200 µg showed the significant adverse effects on cells within 48 h of exposure. Although these NPs do not cause morphological changes in the cells and do not induce apoptosis and necrosis, they can stop the cell cycle and inhibit the cell growth by entering cells (19).

Conclusion

The synthesized iron NPs had adverse effects on the HT-29 cancer cells, which intensified by time passage. Contrary to the bulk particles, NPs induced more

necrosis in HT-29 cells at the lowest level (1.57mM/mL). Noteworthy, the orally iron particles as a bread fortificant in rats did not provoke histopathological lesions in normal intestinal tissue. Since the iron NPs fortified bread had no detrimental effect on the rats' normal intestinal cells, we suggest to study the interaction of iron with bread constituents at a molecular level.

Conflict of Interests

The authors have no conflict of interests in this study.

Ethical Approval

The project underwent ethical review and was approved by the Institutional Animal Care and Use Committee (approval ID: IR.UK.VETMED.REC.1398.006).

Authors Contribution

MB; Contributed to conception and design, responsible for overall supervision, Drafted revised and submitted manuscript. , EM; Contributed to all experimental works, and interpretation of data. HE; supervision of food analysis, Date analysis. , IK; Supervision of histopathological studies., NA; supervision of cell culture studies, All authors read and approved the final manuscript.

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